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Intracellular and Cell-to-Cell Spread of *Listeria monocytogenes* Involves Interaction with F-Actin in the Enterocytelike Cell Line Caco-2

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Listeria monocytogenes **penetrates and multiplies within professional phagocytes and other cells such as the Caco-2 human enterocytelike cell line. Listeriolysin O, a membrane-damaging cytotoxin accounts for intracellular multiplication through lysis of the membrane-bound phagocytic vacuole. This work demonstrates that once released within the cytosol, *L. monocytogenes* acquires the capacity to spread intracellularly and infect adjacent cells by interacting with host cell microfilaments. Such evidence was obtained by using drugs which disrupt the cell cytoskeleton. Nocodazole, which blocks polymerization of microtubules, did not affect intracellular spread, whereas cytochalasin D, which blocks polymerization of G-actin, inhibited the intracellular motility of the bacteria. By using fluorescence staining with 7-nitrobenz-2-oxa-1,3-diazole-phallacidin (NBD-phallacidin), transmission electron microscopy, and immunogold labeling, direct evidence was obtained that intracellular bacteria were enveloped with a thick layer of F-actin. Within 2 h after entry, it was demonstrated by confocal microscopy that bacteria were following highly organized routes corresponding to stress fibers. Four hours after entry, some bacteria presented random movements which could be seen by the presence of a large trail of F-actin. Such movements also caused protrusions which deeply penetrated adjacent cells and resulted in the formation of vacuoles limited by a double membrane. After subsequent lysis of these membranes, bacteria released within the cytoplasm were able to multiply and invade new cells. In contrast, an *hly*::Tn*1545* mutant of the wild-type microorganism demonstrated almost no intracellular spread. Only a few bacteria displaying delayed lysis of the phagocytic vacuole behaved like the wild-type strain. Hemolysin-mediated lysis of the phagocytic vacuole and subsequent interaction with host cell microfilaments may represent a major virulence factor allowing tissue colonization during listeriosis.**

Listeria monocytogenes is a ubiquitous gram-positive, hemolytic, aerobic rod which is motile at room temperature but only slightly at 37°C. It is responsible for severe infections in animals and humans (15). Although various modes of entry have been described, human infections usually follow transplacental (15) or transintestinal (10) routes. Therefore, an essential prerequisite to the establishment of an infection must be the capacity of the microorganism to cross vascular or intestinal barriers. Subsequently, *L. monocytogenes* expresses its virulence by growing within macrophages of the reticuloendothelial system (21). Listeriolysin O, the *L. monocytogenes* hemolysin is a sulfhydryl-dependent protein toxin of 58 kilodaltons (13, 25) which is essential for intracellular growth, as demonstrated in the mouse infection model assay by comparing a wild-type (Hly^+) isolate and its nonhemolytic (Hly^-) derivative created by transposon Tn*1545* mutagenesis (12). Similar results were observed in various cell assay systems which used fibroblasts (18), mouse macrophages (26), or the human enterocytelike cell line Caco-2 (11). Disruption of the membrane-bound phagocytic vacuole correlates with secretion of listeriolysin and intracellular growth (11).

Recent studies of the enteroinvasive pathogen *Shigella flexneri* have shown that, in addition to allowing intracellular growth (28), lysis of the phagocytic vacuole also allows bacteria to spread intracellularly and infect adjacent cells (3). This is an unexpected property for a nonmotile microorganism which has been correlated with *icsA* (*virG*), a gene encoding a 120-kilodalton outer membrane protein which allows interaction with microfilaments within the cell. Intracellularly, *icsA* mutants grow as microcolonies localizing within the cytoplasm. Such mutants demonstrate a dramatic reduction in virulence (3, 22), indicating that the capacity to spread intracellularly and infect adjacent cells is a key virulence determinant. As anticipated, these mutants elicit smaller and more superficial abscesses within the intestinal mucosa of rabbit ligated ileal loops and in orally infected macaque monkeys (27a).

We have established a parallel between the intracellular behavior of *S. flexneri* and that of *L. monocytogenes* and studied whether the latter also has the capacity to move intracellularly. Such movement leading to cell-to-cell spread in murine fibroblasts had already been suggested by Havell (16). Studies carried out to confirm this hypothesis involved the use of a cell assay system in which confluent islets of Caco-2 cells (27) were infected by strain EGD, a hemolytic wild-type isolate which will be called Hly^+, or by its *hly*::Tn*1545* mutant, which will be called Hly^- (12). The Hly^+ strain invaded the periphery of the islets and migrated toward their center. A centripetal movement was deduced from these observations since reinfection of cells during the course of the experiment was made impossible by the addition of gentamicin to the medium. This suggested a capacity to spread intracellularly and infect adjacent cells. In contrast, although invasive, most of the Hly^- bacteria remained localized within the periphery, beneath the cell surface. Whether bacterial movement within the cytoplasm, was dependent upon the routes which exist within a cell (32,

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33) or whether movement was generated independently by the bacteria themselves was analyzed. Bacterial movement was blocked by cytochalasin D, which inhibits polymerization of actin subunits (30), and not by nocodazole, which inhibits polymerization of tubules (20). In addition, an interaction between intracellular bacteria and microfilaments to generate intra- and intercellular movement has been directly demonstrated. A recent study by Tilney and Portnoy (31) carried out with macrophages has demonstrated a similar type of interaction.

MATERIALS AND METHODS

Bacterial strains. Two strains were used in this study: *L. monocytogenes* EGD (Hly^+) and its nonhemolytic derivative (Hly^-), which was obtained by insertion of the transposon Tn*1545* within the hemolysin structural gene (12). Hemolysin production was controlled by growth on 5% horse blood tryptic soy agar. Bacteria were routinely subcultured in tryptic soy broth (Diagnostics Pasteur, Marnes la Coquette, France) for 2 h at 37°C prior to infection of Caco-2 cells to obtain an exponential-phase culture. After being washed in phosphate-buffered saline (PBS), bacteria were suspended in minimum essential medium (MEM) (GIBCO Laboratories, Inc., Grand Island, N.Y.) and used to infect Caco-2 cells.

Culture and infection of Caco-2 cells. The human colonic carcinoma cell line Caco-2 was utilized between passages 76 and 80 (27). Cells were cultured in MEM with 20% fetal calf serum (GIBCO) and 1% nonessential amino acids (Flow Laboratories, Inc., McLean, Va.) in an atmosphere of 5% CO_2 in air. Cells were seeded at a concentration of 2×10^5 cells per ml in culture medium on glass coverslips which were placed into 35-mm plastic tissue culture dishes (Corning Glass Works, Corning, N.Y.). Semiconfluent monolayers obtained after a 48-h incubation period were washed once with Earle balanced salt solution (GIBCO) before being infected. At this stage, cells formed roughly circular islets composed of 10 to 20 confluent cells. Monolayers were inoculated with a bacterial suspension in MEM to obtain a multiplicity of infection of 10 bacteria per cell. Contact between bacteria and cells was facilitated by centrifugation (2,000 × *g*, 10 min) at 20°C and incubation for 1 h at 37°C. After three washes with Earle balanced salt solution, the medium was supplemented with gentamicin (5 μg/ml) in order to kill extracellular bacteria, thus preventing further reinfection of cells, and incubated for additional 2- and 4-h periods. At these points, cells were washed and fixed for fluorescence staining.

Treatment of infected Caco-2 cells with drugs which interfere with polymerization of cytoskeleton subunits (20, 30). Following a 45-min infection period Caco-2 cells were treated either with cytochalasin D (Sigma Chemical Co., St Louis, Mo.) at a final concentration of 0.5 μg/ml in MEM and gentamicin or with nocodazole (12 μM); treatments were done 2 h prior to infection and over the incubation period.

Fluorescence staining of F-actin and bacteria. Permeabilization and cell fixation have been previously described (6). After three washes in PBS and one in PHEM [10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM $MgCl_2$, 60 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 23 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH 6.9], infected cells were permeabilized for 1 min in 0.5% Triton X-100 in PHEM. Fixation was achieved by a 3-min incubation in acetone at −20°C. Bacteria were then labeled by indirect immunofluorescence. After drying, coverslips were washed in PBS with 0.2% gelatin, incubated in a 1/100 dilution of a rabbit antiserum raised against *L. monocytogenes*, and revealed by incubation in a 1/80 dilution of goat anti-rabbit rhodamine-conjugated immunoglobulin G (Sigma) for 20 min. For staining of F-actin, coverslips were incubated in a 10-U/ml solution of 7-nitrobenz-2-oxa-1,3-diazole-phallacidin (NBD-phallacidin) (Molecular Probes, Inc., Junction City, Oreg.) for 20 min (1).

Fluorescence staining of tubulin and bacteria. Bacteria were labeled as described above and revealed by incubation in a 1/80 dilution of a goat anti-rabbit fluorescein-conjugated immunoglobulin G (Sigma). Tubulin was stained by indirect immunofluorescence. An antitubulin serum (Amersham Corp., Buckinghamshire, England) diluted 1/300 and a goat anti-mouse rhodamine-conjugated immunoglobulin G (Sigma) diluted 1/200 were successively used. More than 200 cells were observed in each preparation. On the average, the features described in this paper were present at variable degrees in more than 50% of the cells observed.

Confocal microscopy. Fluorescent preparations were observed in a confocal fluorescence imaging system, using an MRC-500 Lasersharp (Bio-Rad, Abington, Oxfordshire, England). Pictures were recorded on a flat-screen black and white monitor with high linearity. This technique, which allows observation of cell structures lying in a single plane, eliminates the diffuse fluorescent background present with conventional methods and demonstrates colocalization of adjacent structures.

Electron microscopy. Following a 48-h growth period, cell monolayers were infected with 20 bacteria per cell (Hly^+) and 40 bacteria per cell (Hly^-) by the procedure described above. Infected cells were fixed for 1 h at 20°C with a mixture of 2.5% glutaraldehyde and 2.5% paraformaldehyde in cacodylate buffer (pH 7.2) containing 0.1 M sucrose. They were washed in the same buffer, postfixed for 1 h with 1% osmium tetroxide, scraped off the culture dishes, concentrated in agar by centrifugation, and treated for 1 h with 1% uranyl acetate dissolved in Veronal buffer (pH 6). They were then dehydrated in acetone and embedded in Epon.

For immunogold labeling, cells were fixed for 1 h at 4°C with 2.5% paraformaldehyde–0.1% glutaraldehyde in cacodylate buffer. After two washes, they were incubated for 30 min at 4°C in cacodylate buffer containing 50 mM ammonium chloride. They were then scraped off the culture dishes, concentrated in agar, and treated for 30 min with 0.5% uranyl acetate to improve the sectioning properties of the bacteria and to preserve the ultrastructure of the host cells (J. C. Bénichou, C. Frehel, and A. Ryter, J. Electron Microsc. Tech., in press). Finally the cells were dehydrated in ethanol and embedded in Lowicryl K4M at −25°C (35). Lowicryl thin sections were collected on nickel grids and incubated for 1 h with an actin-specific monoclonal antibody (4) diluted in PBS containing 1% bovine serum albumin. They were washed several times in PBS–0.5% bovine serum albumin and incubated for 1 h with a gold anti-mouse immunoglobulin conjugate diluted in PBS–1% bovine serum albumin. After being washed, thin sections were stained with uranyl acetate and lead citrate. Controls were performed with two monoclonal antibodies which react with two antigens absent in eucaryotic cells. The nonspecific adsorption of the gold conjugate was determined by incubating thin sections with the gold conjugate without previous incubation with the antibody.

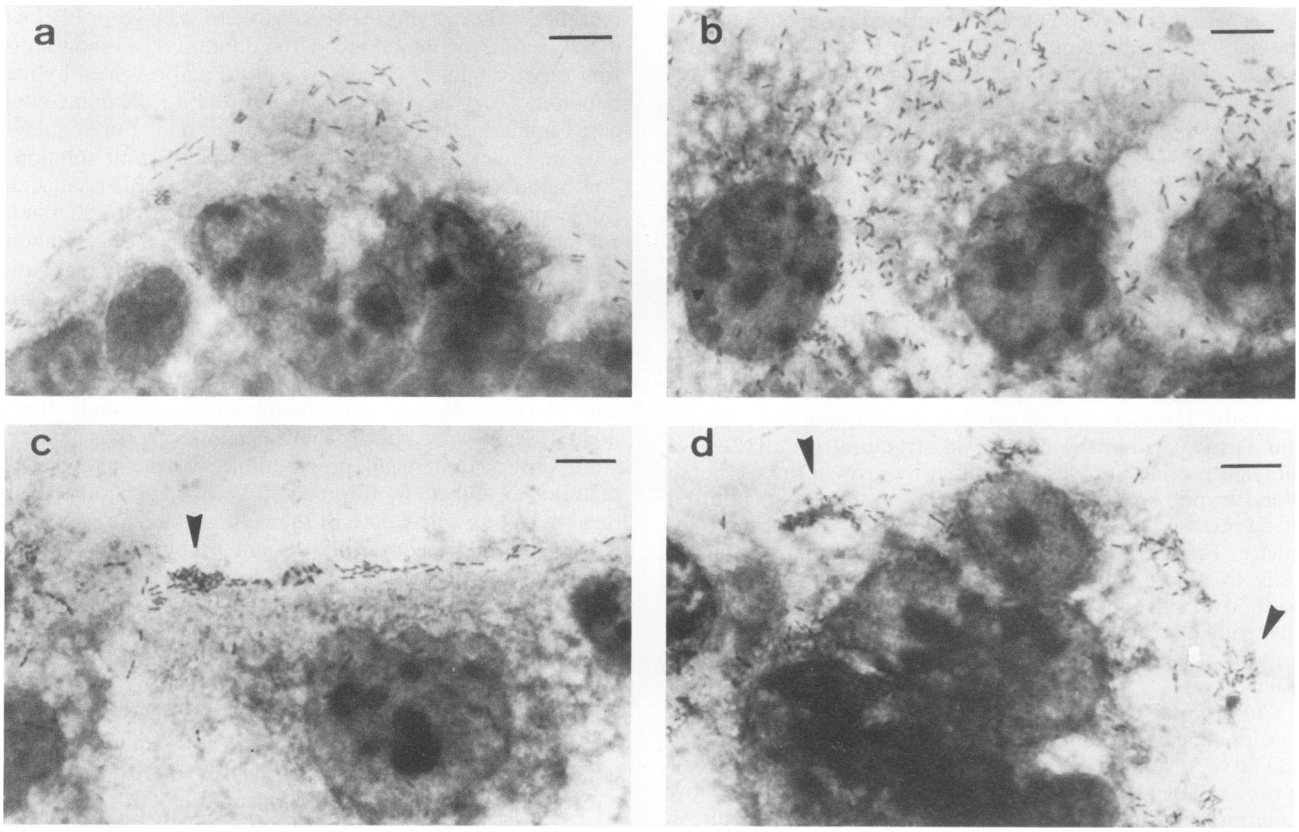


FIG. 1. Intracellular and cell-to-cell spread of *L. monocytogenes* EGD (Hly^+) and its Hly^- mutant within Caco-2 cell islets. Giemsa staining was used. (a and b) Hly^+ strain after 2 and 4 h of incubation, respectively, in the presence of gentamicin; (c and d) Hly^- strain after 2 and 4 h of incubation, respectively, in the presence of gentamicin. Arrowheads point to areas were bacteria localized. Bars = 10 μm.

RESULTS

Intracellular movement of *L. monocytogenes* EGD (Hly^+) and its nonhemolytic mutant (Hly^-). After infection of islets of Caco-2 cells by *L. monocytogenes* EGD, early observations made following a 2-h incubation (Fig. 1a and c) demonstrated that invading bacteria were located underneath the cell surface, at the periphery of the islets. Interestingly, the entry process occurred on the outer edge of the peripheral cells, suggesting that the low level of differentiation of such cells and the abundance of actin filaments in the areas of cell adherence to coverslips may play a considerable role in facilitating entry in these zones. It must be emphasized at this point that this cell assay system did not make use of the polarity expressed by Caco-2 cells when grown at complete confluency. Transmission electron microscopy confirmed early lysis of the phagocytic membrane, as has previously been demonstrated (see Fig. 4a and b); this will be further discussed below (11). Centripetal colonization of islets, as illustrated in Fig. 1a and b, could be observed after 2 and 4 h of incubation, respectively. These data suggested that *L. monocytogenes* was capable of intracellular and cell-to-cell spread at 37°C, since extracellular bacteria were killed by gentamicin.

Islets of Caco-2 cells were also infected by the Hly^- mutant (12). Observations made after 2 and 4 h of infection showed that invading bacteria remained localized at the periphery of the islets, essentially underneath the cell surface. Unlike the Hly^+ strain, no Hly^- bacteria had spread intracellularly after 2 h of incubation (Fig. 1c) and only a very limited number of them had spread after 4 h of incubation (Fig. 1d). Most of the invading microorganisms remained localized near their sites of entry. Transmission electron microscopic observations which are described below (see Fig. 5c) demonstrated that, unlike the wild-type microorganism, most of these intracellular bacteria (i.e., >90% of the intracellular bacteria observed) remained surrounded by an intact phagocytic membrane, as previously observed (11). These experiments demonstrated that failure to produce listeriolysin O dramatically decreased the capacity of *L. monocytogenes* to spread intracellularly and infect adjacent cells.

Intracellular movement and cell-to-cell spread of *L. monocytogenes*. In order to demonstrate whether *L. monocytogenes* moved passively within cells or used cellular structures that support movement, such as microtubules or microfilaments (32, 33), we treated cells with nocodazole, which blocks polymerization of tubulin subunits (20), and cytochalasin D, which blocks polymerization of G-actin (30). Disruption of cell microtubules by nocodazole did not affect the spread of strain EGD (Fig. 2g). Controls demonstrated complete disruption of microtubules of Caco-2 cells at the concentration (12 mM) which was utilized here (data not shown). Double labeling of bacteria and microtubules in cells which had not been treated by nocodazole did not demonstrate colocalization (Fig. 2h). This suggested that intracellular movement of *L. monocytogenes* does not require microtubules. In contrast, destruction of cell microfilaments by cytochalasin D interrupted both intracellular and cell-

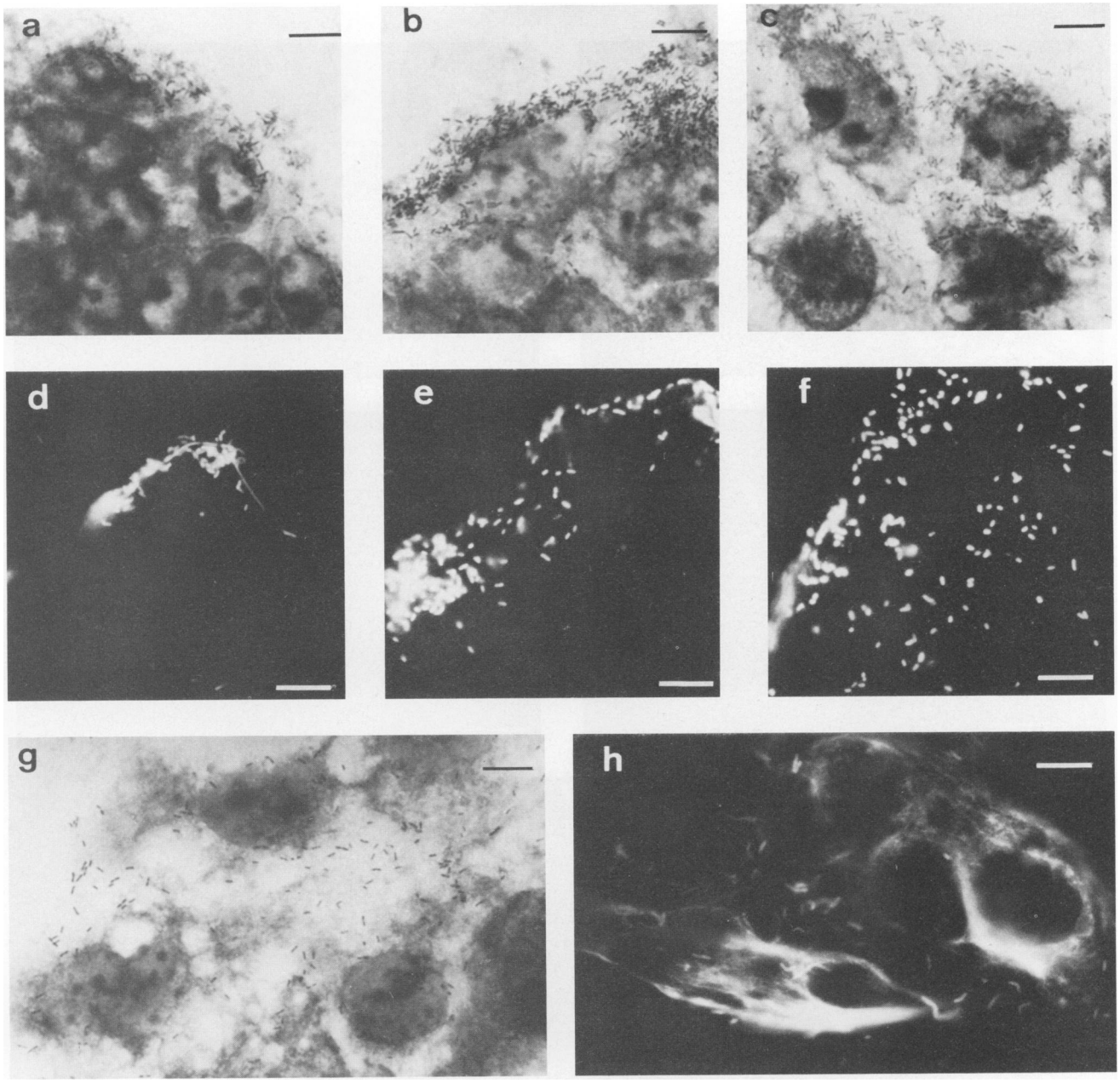
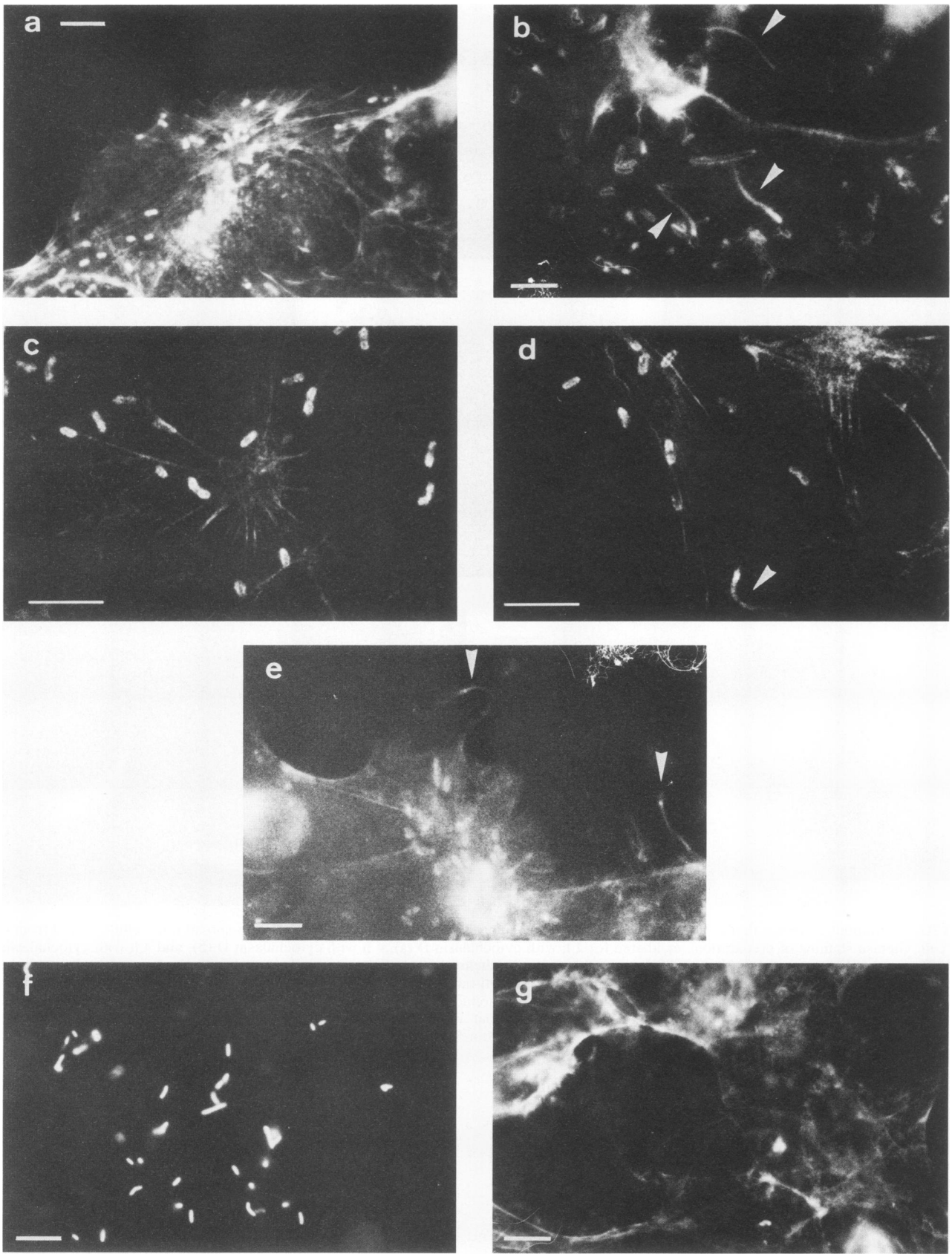


FIG. 2. Indirect evidence that *L. monocytogenes* EGD uses microfilaments and not microtubules to spread intracellularly and from cell to cell. Giemsa staining of preparations incubated for 2 h with cytochalasin D (a), 4 h with cytochalasin D (b), and 4 h with cytochalasin D 1 h after removal of the drug (c). (d, e, and f) Immunofluorescence labeling of bacteria treated as for panels a to c, respectively. Bacteria were labeled by using a rabbit anti-*L. monocytogenes* serum and a goat anti-rabbit, rhodamine-conjugated, immunoglobulin G. (g) Giemsa staining, 4 h of incubation in the presence of nocodazole; (h) double immunofluorescence labeling, cells not treated with nocodazole, microtubules labeled by using an antitubulin mouse monoclonal antibody and goat anti-mouse rhodamine-conjugated immunoglobulin G (bacteria were labeled as described above except that the anti-rabbit serum was a fluorescein isothiocyanate-labeled goat immunoglobulin G. Bars = 10 μm.

to-cell spread of strain EGD (Fig. 2a to f). After 2 and 4 h of incubation in the presence of this drug, intracellular bacteria localized underneath the cell surface and grew as microcolonies (Fig. 2a, b, d, and e). Thirty minutes after removal of the drug, movement of bacteria resumed within cells, leading to colonization of the islet (Fig. 2c and f). This observation indicated that EGD uses host cell microfilaments to move intracellularly.

Involvement of F-actin in the spread of strain EGD was demonstrated by labeling Caco-2 cells with NBD-phallacidin after various periods of incubation. Two periods could be distinguished (Fig. 3). After 2 h (Fig. 3a), intracellular bacteria appeared brightly labeled by the reagent, indicating that they were heavily covered with F-actin. In addition, they could be observed only in areas of the cells which were rich in actin cables, suggesting that they might move along stress fibers for initial intracellular spread. This hypothesis was supported by observations made by confocal microscopy (Fig. 3c), which showed perfect colocalization of bacteria and stress fibers. These findings suggested that


a
b
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bacteria attached to stress fibers via their F-actin-coated surfaces.

After 4 h of infection (Fig. 3b), some bacteria appeared to leave the environment of stress fibers and started to generate random movements which could be seen by the appearance of F-actin trails following bacteria which remained covered with F-actin. The diameter of the trails was equal to that of labeled bacteria. Confocal microscopy confirmed that among the numerous bacteria interacting with stress fibers, one bacterium generated random movement indicated by a thick trail of F-actin (Fig. 3d). Transmission electron microscopy and immunogold labeling data which are presented below confirmed these data. It is not known as yet whether coats and trails were exclusively composed of F-actin or contained other components, particularly gel- or bundle-forming actin-binding proteins (29). Double fluorescence labeling using NBD-phallacidin and an antimyosin polyclonal rabbit serum labeled with rhodamine (data not shown) did not suggest the presence of significant amounts of myosin within F-actin coats and trails.

Observation of the various fluorescent preparations after 2 h of infection did not yield evidence of cell-to-cell spread. However, after 4 h of infection, movements led to the formation of membrane protrusions, internally labeled by NBD-phallacidin, which contained a bacterium at their tip (Fig. 3e). Thus, intracellular movements of bacteria could cause the formation of protrusions in infected cells. Involvement of these protrusions in infection of adjacent cells was demonstrated by analysis of multiple sections by transmission electron microscopy (see below).

On the other hand, double fluorescence labeling of cells infected by the Hly^- mutant after 2 h of incubation did not show evidence of labeling with NBD-phallacidin (Fig. 3f and g), suggesting that the bacterial surface had no direct contact with host cell microfilaments.

Transmission electron microscopic analysis of intracellular movement and cell-to-cell spread. Examination of cells infected by strain EGD showed that 2 h after infection, occasional bacteria still appeared to be enclosed within a membrane-bound vacuole (Fig. 4a), whereas the large majority appeared free within the cytoplasm (Fig. 4b). Soon after phagosomal lysis, bacteria appeared in close contact with the cytoplasmic matrix and cell organelles (Fig. 4b). Such bacteria were rapidly surrounded by a thick layer of dense spongy material which excluded cytoplasmic constituents (Fig. 4c). After 4 h of incubation, many bacteria could be found within protrusions budding from the cell surface (Fig. 4d). Some penetrated deeply into adjacent cells by deforming their plasma membranes (Fig. 4e and f). They finally appeared to form elongated vacuoles filled with loose spongy material and limited by a double membrane (Fig. 5a). The double membrane consisted of the apposition of the membrane of the protrusion and that of the penetrated adjacent cell. Figure 5b suggests that bacteria enclosed within these double membrane-bound vacuoles finally lysed them and became free within the cytoplasm. Release probably allowed multiplication and subsequent movement of the bacterium within the newly infected cell, thus permitting infection of new adjacent cells and so on.

Experiments using immunogold labeling were performed on Lowicryl K4M thin sections with monoclonal antibodies specific for G- and F-actin (4), which were subsequently detected with a gold-anti-mouse immunoglobulin conjugate. These experiments confirmed observations made after staining of infected cells with NBD-phallacidin. Figure 6a and b show gold particles concentrated on the spongy material accumulated around the bacterium. A few gold particles were dispersed within the cytoplasm and were present in greater density underneath the plasma membrane (Fig. 6a). In addition, Fig. 6c shows the transverse section of a protrusion which appears filled with a spongy material intensely labeled with gold particles. Control experiments were performed in which gold-anti mouse immunoglobulin complex and the two monoclonal antibodies that recognize noneucaryotic antigens were used. No significant labeling with gold particles could be observed.

Observation of cells infected by the Hly^- mutant showed that all bacteria remained enclosed within phagosomes after 2 h of incubation. After 4 h, many phagosomes had fused to form larger pockets in which dense lysosomal material could be observed (Fig. 5c). Rare bacteria, however, were seen free within the cytoplasm surrounded by a thick layer of actin. These bacteria may correspond to the very small subset which could be seen colonizing the cell islet. On the other hand, accumulation of F-actin was never observed around intact phagosomal vacuoles.

DISCUSSION

Several bacterial species have the capacity to invade eucaryotic cells (24). Available data indicate that entry strategies evolved by these bacteria are quite different (2, 17, 19), although it is likely that, as shown for *S. flexneri* (6), these microorganisms first enter cells through directed phagocytosis. Once intracellular, bacteria demonstrate two distinct characteristics. Some invasive organisms like *Salmonella* or *Yersinia* species remain entrapped within a membrane bound-phagocytic vacuole (8, 23, 28). The intraphagosomal environment, which is even more inhospitable once phagolysosomal fusion occurs, has necessitated the development of a series of mechanisms in order to allow survival and multiplication. Entrapped bacteria may have to follow the routes utilized by intracellular organelles, as recently suggested for polarized MDCK cells infected by *Salmonella choleraesuis* (9). This microorganism appears to transcytose from the apical to the basal pole of these cells. The driving force of this polarized movement has not yet been characterized. Other invasive organisms like *Shigella* (28) and *Rickettsia* (36) species and *L. monocytogenes* have the capacity to induce rapid lysis of the membrane-bound phagocytic vacuole. Membrane-damaging properties, although different in nature, are expressed by each of these different species. *S. flexneri* expresses a complex surface hemolytic activity also necessary to trigger the entry process

FIG. 3. (a–e) Direct evidence that *L. monocytogenes* EGD uses microfilaments to spread intracellularly and from cell to cell. Labeling was with NBD-phallacidin only. (a) Two hours of incubation, bacteria labeled by NBD-phallacidin are concentrated in areas rich in stress fibers. (b) Four hours of incubation, bacteria are labeled by NBD-phallacidin and in several instances (arrowheads) are followed by a trail containing F-actin. (c and d) Confocal microscopy, bacteria labeled by NBD-phallacidin are seen interacting with stress fibers at 2 and 4 h of incubation, respectively; after 4 h of incubation, some bacteria (as in panel b) generated actin trails (arrowhead). (e) Four hours of incubation, arrowheads point to membrane protrusions with an F-actin-coated bacterium at its tip. (f and g) Double fluorescence labeling of bacteria (f) and F-actin (g) after 2 h of incubation. None of the intracellular bacteria are labeled with NBD-phallacidin (compared with panel a). Bars = 10 μm.

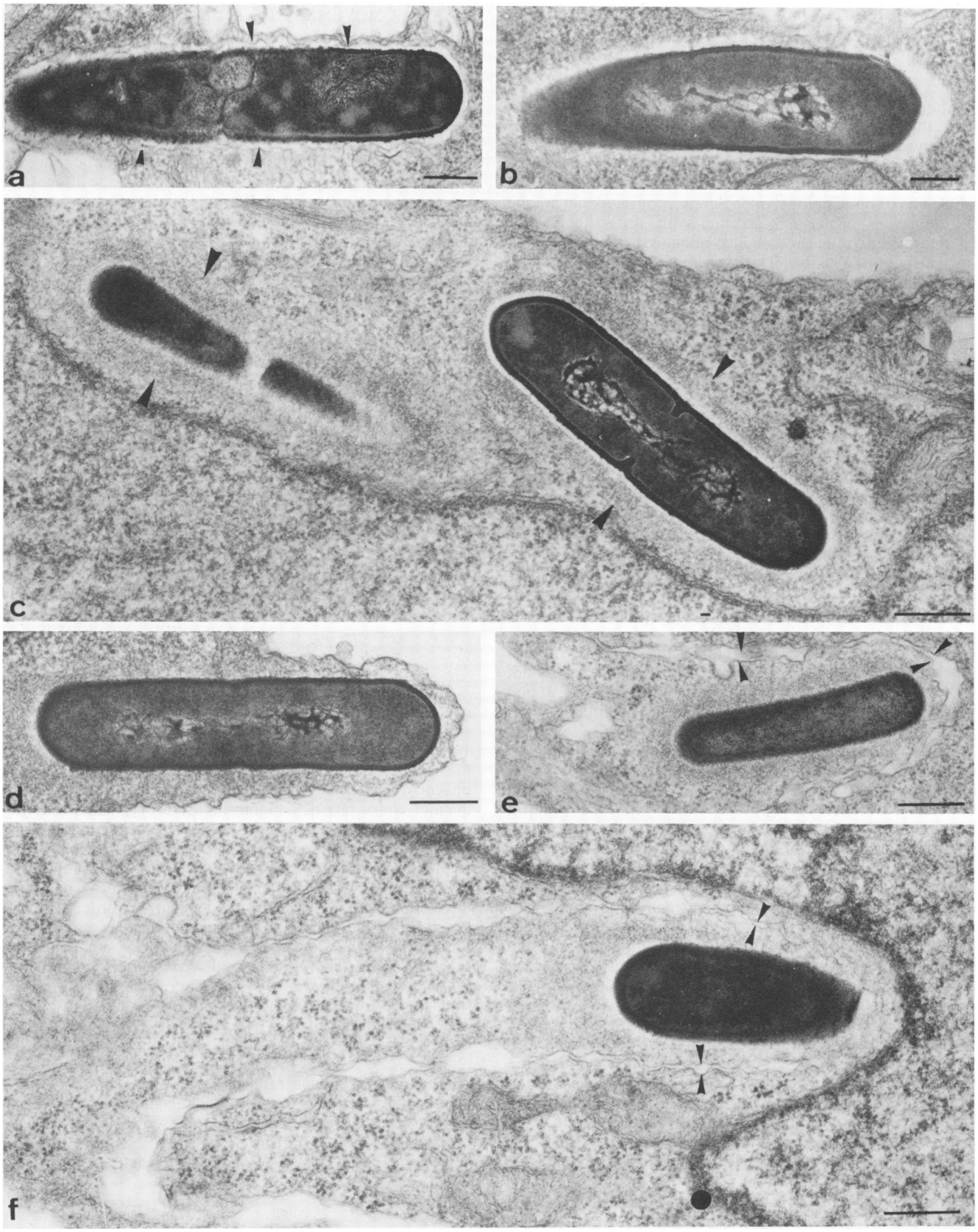

FIG. 4. Thin sections of Caco-2 cells infected for 1 h with *L. monocytogenes* EGD and reincubated in fresh medium for 2 h (a, b, and c) or 4 h (d, e, and f). (a) The phagosome membrane (arrowheads) is still present around the bacterium. (b) The phagosome membrane has been destroyed, and the bacterium appears in direct contact with the cytoplasmic matrix and cell organelles. (c) Bacteria are surrounded by a thick layer of dense spongy material (arrowheads). (d) Some of the bacteria surrounded by spongy material from a protrusion budding on the cell surface. (e and f) Protrusions penetrating adjacent cells; the membrane of the protrusion and the plasma membrane of the adjacent cell are both visible (arrowheads). Bars = 0.5 μm.

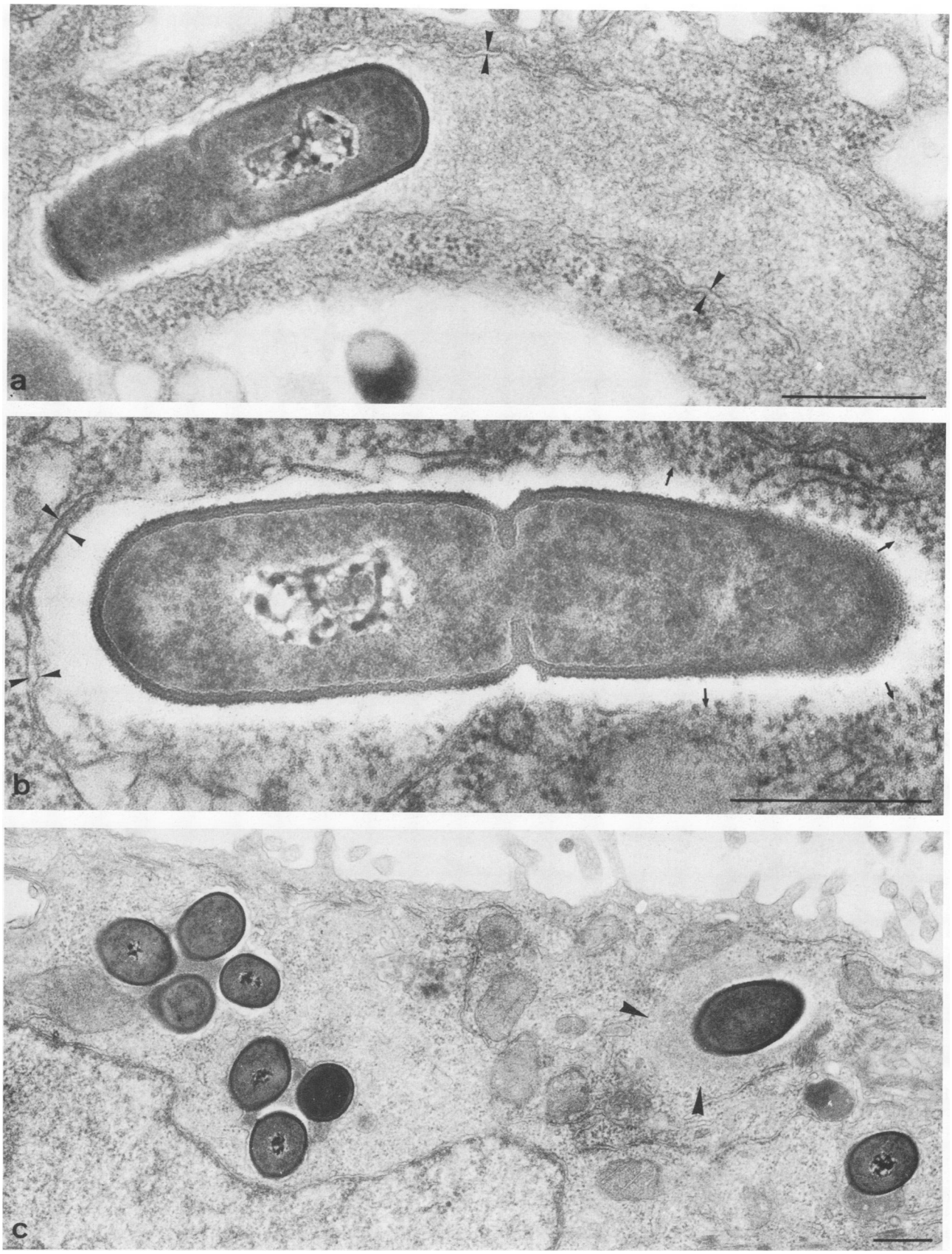

FIG. 5. Sections of Caco-2 cells infected with *L. monocytogenes* Hly^+ or Hly^-. (a) Four hours of incubation; example of a protrusion located in the cytoplasm of an adjacent cell. Protrusion is limited by a double membrane (arrowheads). (b) Ongoing destruction of a double membrane surrounding a bacterium. On the left (arrowheads), both membranes are still visible, whereas on the right (arrows), they have already disappeared. Bars = 0.5 μm. (c) Four hours of incubation after infection with *L. monocytogenes* Hly^-. Most bacteria are located in large phagosomes in which dense lysosomal material is visible. One bacterium located on the right has dissolved the phagosome membrane and is surrounded by a thick layer of dense spongy material (arrowheads). Bar = 10 μm.

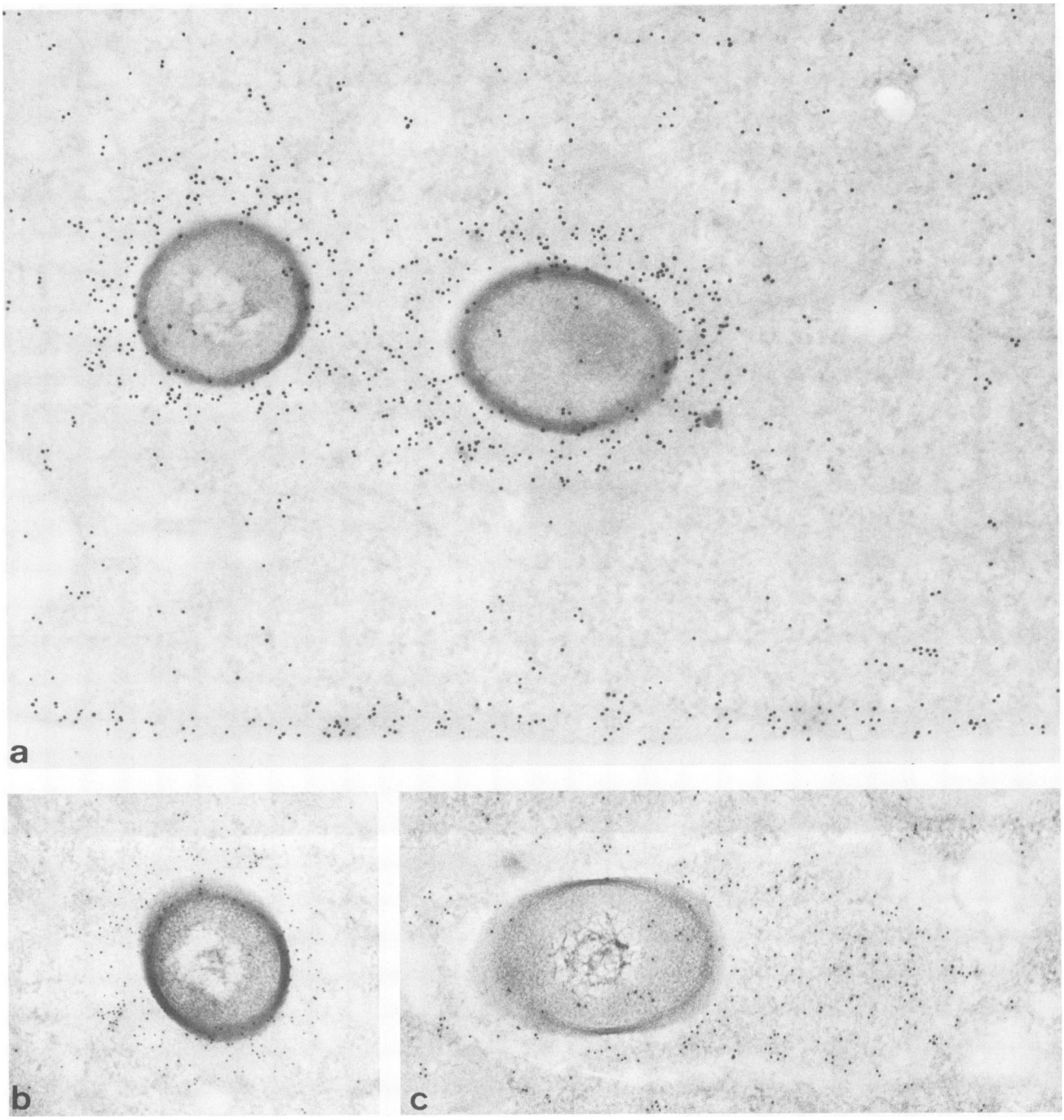

FIG. 6. Immunogold labeling performed on Lowicryl thin sections of Caco-2 cells infected with *L. monocytogenes* EGD and subsequently incubated for 3 h. (a and b) Gold particles are concentrated around the bacterium. In panel b, they are also abundant along the plasma membrane and more dispersed in the cytoplasm. (c) Protrusion showing strong labeling on the spongy material surrounding the bacterium.

(5, 28), rickettsiae produce a phospholipase A_2 (36), and *L. monocytogenes* produces listeriolysin O, which accounts for lysis of the phagocytic vacuole and intracellular growth (11). Release from the phagocytic vacuole allows bacteria to scavenge nutrients necessary for intracellular growth and to escape phagolysosomal fusion. In addition, this property provides them with the potential to interact with components of the cell cytoskeleton which allow intracellular movement and cell-to-cell spread, as described in the present work. Intracellular movement and cell-to-cell spread is now an established phenomenon in *S. flexneri* (3, 22), *Rickettsia tsutsugamushi* (7), and *L. monocytogenes* (16, 31).

The relevance of intracellular movement and cell-to-cell spread to bacterial pathogenicity may be questioned. There is no doubt that it is a major virulence factor for *S. flexneri*. The *icsA* (*virG*) mutant, which no longer expresses a 120-kilodalton outer membrane protein that interacts with microfilaments to generate intra- and intercellular spread, is negative in the guinea pig keratoconjunctivitis assay (3, 22). It causes only small abscesses at the tip of intestinal villi in the

rabbit ligated ileal loop invasion assay and demonstrates considerably reduced invasiveness when fed orally to macaque monkeys (27a). The importance of this phenotype in the development of abscesses may be even more crucial if, as recently suggested, *S. flexneri* invades the intestinal mucosa via M cells (34). Intra- and intercellular spread would therefore appear to be the major virulence phenotype allowing "underground" colonization of epithelia.

As elegantly shown recently by Tilney and Portnoy in J774 macrophages (31), *L. monocytogenes* spreads both intracellularly and from cell to cell. The relevance of this phenotype in the virulence of this species has yet to be assessed. Construction of a mutant which does not spread either intra- or intercellularly will be the next step. One can speculate that it is necessary for the invasive microorganism to pass barriers which would otherwise impair development of the infection process: intestinal epithelial cells or endothelial cells at the level of the blood-brain barrier or of the placenta. It may also be essential for the infection of adjacent cells within infection foci of the reticuloendothelial system leading to the development of abcesses.

We have eliminated the possibility that the movement of *L. monocytogenes* within cells is related to a microtubule-based transport system in which the bacteria use or produce equivalents of energy-generating enzymes such as dynein (14) or kinesins (32). Pretreatment of infected Caco-2 cells with nocodazole, although causing total depolymerization of microtubules, did not affect the movement of strain EGD at all. Concerning the very small subset of Hly^- bacteria which moved intracellularly, lysed their phagocytic vacuole, and accumulated F-actin on their surfaces after 4 h of incubation, it is suggested that in the absence of production of listeriolysin O, the *L. monocytogenes* phospholipase C accounts for late lysis of the vacuole. Only the obtention and use of a mutant could answer this question.

Two steps were shown to occur after lysis of the phagocytic vacuole. Bacteria became covered with F-actin soon after destruction of the phagosomal membrane, as demonstrated by both fluorescence and immunoelectron microscopy. Concomitantly, bacteria appeared to interact with actin cables that they might use to spread intracellularly in an organized movement. In a second step, bacteria appeared to deviate from the F-actin trails and generate random movements evidenced by the presence of F-actin trails following such bacteria. The latter type of movement resulted in the formation of protrusions through which bacteria could penetrate adjacent cells. This is perfectly consistent with the observations made by Tilney and Portnoy with macrophages (31). Finally, lysis of the double membrane surrounding the bacterium allowed bacterial multiplication, as well as induction of a new cycle of infection and cell-to-cell spread.

Besides the involvement of F-actin, the mechanical bases of bacterial movement are as yet unclear. It is generally considered that myosin is the motor of actin-based movement (33). No direct evidence of involvement of myosin has been demonstrated in this system (data not shown). Work is in progress to demonstrate whether F-actin-associated movement of *L. monocytogenes* is due to the presence of a myosinlike procaryotic surface protein. However, in *S. flexneri* (3), no evidence of myosin accumulation has been observed either. We believe that either de novo polymerization of G-actin and/or brisk gelation of F-actin accounts for this property. Whether the bacterium expresses an actin-binding-like protein or a protein which interacts with an actin-binding protein is as yet unknown. Alternatively, changes in the metabolic conditions surrounding the bacterium (i.e., Ca^{2+} concentration and pH) may precipitate an actin gel in the vicinity via rapid gelation or bundling of F-actin, thus propelling the bacillus forward.

Future work will be aimed at characterizing the gene(s) and gene product(s) responsible for interaction of *L. monocytogenes* with the cytoskeleton of infected Caco-2 cells and at better defining the various components of the F-actin substrate which accumulate around and cause spreading of the bacteria.

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